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14. ABSTRACT

This research project investigates the lipid content and composition of extremophilic and estuarine microalgae under different growth conditions for suitability as biofuel feedstocks. Fatty acid content and lipid composition are major considerations in optimizing algae as feedstocks for fuel production. In this project fatty acid-methyl ester analysis (FAME GC-MS) was used to characterize the acyl content of the typical feedstock alga *Chlorella saccharophila*, the unusual acidothermophilic alga *Galdieria sulphuraria*, and cold-tolerant primary cultures cultured from the Severn River through winter bioprospecting. The cold tolerant species' acyl content included a greater percentage of C16 and highly unsaturated hydrocarbon chains, while *Galdieria* had a greater percentage of C18 and less unsaturated hydrocarbon chains, in comparison to *Chlorella*. In addition, a new, high-recovery method of direct FAME extraction from algal biomass was used to examine fatty acid content and composition from *Galdieria* and *Chlorella* cultures grown under a variety of nutrient conditions, including mixotrophic and autotrophic growth. *Chlorella* demonstrated a typical and expected effect of nutrient variation on algal lipid production. In contrast, *Galdieria* produced a greater percentage of fatty acids when grown with sugar, even while achieving higher culture density. This unusual effect of mixotrophic growth on *Galdieria* suggests it has potential to be beneficial as a biofuels feedstock, as this organism may alleviate the need to choose between high cell growth and high fatty acid percentages in algal culture.

15. SUBJECT TERMS
algae, biofuel, biodiesel, fatty acid methyl ester, extremophile, *Galdieria*

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CHARACTERIZATION OF MICROALGAL LIPIDS FOR OPTIMIZATION OF BIOFUELS

by

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ABSTRACT

This research project investigates the lipid content and composition of extremophilic and estuarine microalgae under different growth conditions for suitability as biofuel feedstocks. Fatty acid content and lipid composition are major considerations in optimizing algae as feedstocks for fuel production as they determine important characteristics including melting point, flashpoint, cetane number, and fuel value. In this project fatty acid-methyl ester analysis (FAME GC-MS) was used to characterize the acyl content of the typical feedstock alga *Chlorella saccharophila,* the unusual acidothermophilic alga *Galdieria sulphuraria*, and cold-tolerant primary cultures cultured from the Severn River through winter bioprospecting (ENS Gehlhausen SC495/496 project, AY2012). The cold tolerant species' acyl content included a greater percentage of C16 and highly unsaturated hydrocarbon chains, while *Galdieria* had a greater percentage of C18 and less unsaturated hydrocarbon chains, in comparison to *Chlorella*.

In addition to these investigations of cold and heat-tolerant algal lipids, a new, high-recovery method of direct FAME extraction from algal biomass was used to examine fatty acid content and composition from *Galdieria* and *Chlorella* cultures grown under a variety of nutrient conditions, including mixotrophic and autotrophic growth and growth with varying amounts of nitrogen. *Chlorella* demonstrated a typical and expected effect of nutrient variation on algal lipid production: the addition of sugar significantly boosted growth but decreased fatty acid yield as percent of total mass. In contrast, the results of this study suggest that *Galdieria* produced a greater percentage of fatty acids when grown with sugar, even while achieving higher culture density. This unusual effect of mixotrophic growth on *Galdieria* suggests it has potential to be beneficial as a biofuels feedstock, as this organism may alleviate the need to choose between high cell growth and high fatty acid percentages in algal culture.

KEYWORDS

algae, biofuel, biodiesel, fatty acid methyl ester, extremophile, *Galdieria*

ACKNOWLEDGEMENTS

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BACKGROUND INFORMATION

Most biofuels can be categorized as biodiesel products (to include biodistillates) or bioethanol. Corn and sugar cane undergo fermentation in order to produce bioethanol which is a gasoline additive that improves emissions and increases motor performance. The focus of this project will be on biodiesel products. Vegetable oils, algal oils, and animal fats undergo transesterification in order to produce pure diesel fuel or diesel additives (1).

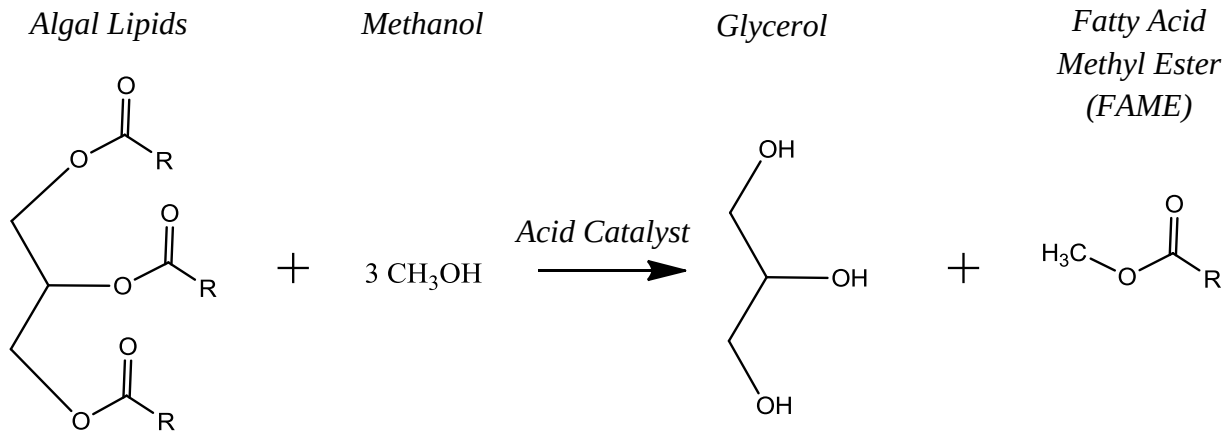
The market for algal biofuels is rapidly expanding. After failed attempts between the late 1970s and 1990s, business for algae biofuels flourished in the 21st century due to technological advancements (1). Companies started "using synthetic biology techniques, along with other biotech and engineering advances, to bring costs down, making algae more efficient by changing the way the organisms use light, increasing the oil content of cells, and improving their efficiency at producing fuel precursors" (1). Fast-growing algae are competitive as an alternative source of fuel because corn and sugar-based ethanol "have only about two-thirds of the energy content as the same volume of gasoline [and] the alcohol isn't well suited for fueling aircraft and heavy trucks" (1). Additionally, fuel additives are necessary for corn and sugar-based ethanol to produce the same amount of energy as algal lipids produce. Algae farms have been able to produce "between 9353 and 60,787 liters per hectare per year of fuel" (1). In comparison, corn and sugar cane required more land and water resources to produce the same volume of fuel. In order to further increase algal fuel density, companies are trying to optimize growing conditions for the algae. Some strains of algae can grow in brackish water, salt-water, or even waste treatment water which greatly increases the amount of available land that can be used for algae farming. Multiple green design approaches for growth include open-air, shallow ponds as well as enclosed chambers called bioreactors. While ponds do not require significant funds to build and maintain, the algal yield is lower than the yield gained from more costly bioreactors (1).

In comparison to algal biofuels, petroleum produces a greater range of fuel products. Petroleum consists of a variety of hydrocarbons; most are classified as alkanes, cycloalkanes, or aromatic hydrocarbons. A hydrocarbon is a series of carbons bonded together with hydrogens bonded to the carbons. These chains can contain anywhere from one carbon to more than 20 carbons. Fractional distillation separates these hydrocarbons based on their volatilities. The fraction containing C12 (a hydrocarbon with 12 carbons) to C18 hydrocarbons is typically used for diesel fuel (2). In contrast, the "unrefined" composition of algal biofuel consists of lipids esterified with saturated and *cis*-unsaturated hydrocarbon chains typically between 14 to 18 carbons in length. Unsaturation occurs through a dehydrogenation reaction which removes two hydrogens, leaving a double-bonded carbon pair.

The process of converting algal lipids into biodiesel products is called transesterification. The majority of algal lipids are triglycerides originating from cell vacuoles or free droplet oil or phospholipids which compose the cell membrane. These lipids, once extracted from the cell, undergo transesterification in order to produce fatty acid methyl esters (FAMEs). **Reaction 1** details this reaction. Fatty acid methyl esters are biodiesel products that can be placed directly in a modern diesel engine with only minor engine modifications such as solvent resistant-seals (3).

The R group designates a fatty acid residue. The hydrocarbons in biofuels are attached to a carbon backbone structure through an ester linkage that must be broken in order to use these fatty acids as biodiesel. These chains can differ in length and in number of unsaturations.

Reaction 1. Fatty Acid Methyl Ester (FAME) Transesterification Reaction



Biodiesel fuel properties are highly dependent on the fatty acid content of the algae providing yet another optimization condition for scientists. These properties include cetane number, melting point, fuel value, and viscosity, to name a few. The focus of this project is to improve algal biofuel feedstocks through discovery of organisms with a more favorable lipid profile and/or content; identifying an algal species that has favorable fatty acids will generate a more effective biofuel requiring fewer additives (4). In general, with increased chain length and increased saturation, the cetane number, fuel value, melting point, and viscosity increase. Ideally, a low melting point is desired because the lower the melting point, the less engineering effort required to maintain the fuel in the liquid state. Increased *cis* unsaturation lowers the melting point of the fatty acid by disrupting hydrophobic packing. (*cis*-unsaturations are double bonds between two carbons on the same side of the double bond in comparison to *trans*-unsaturations where the carbons are on either side of the double bond.) Therefore, a fatty acid with a high degree of *cis* unsaturation leads to a more efficient biofuel (4).

Extremophilic algae hold high potential for optimized engineering of biofuel feedstock production according to biofuels expert Rene H. Wijffels. Wijffels' argument centers on an extremophile's ability to grow "under extreme conditions such as high or low pH, high temperatures, or high salinity" (5). It is hypothesized that extremophilic species may also utilize novel fatty acid profiles which could enhance biofuels properties. There are two categories of extremophilic algae that will be explored through this experiment. The first will be heat-tolerant species such as *Galdieria sulphuraria,* found in hot springs (6). Heat-tolerant species may be better-suited for bioreactors than organisms such as *Chlorella* that thrive at ambient temperature

(7). For bioreactors high powered bulbs provide synthetic light for the algae, and these bulbs consequently heat up the algae growth chambers. If the bioreactor heats up beyond the favorable growth conditions for the *Chlorella* organism, energy and resources must be used to cool down the bioreactor, increasing the input cost. However, a species such as *Galdieria* that prefers hot temperatures would not require the growth chamber to be cooled (8).

Furthermore, there may be some uses for fuels derived from a cold-tolerant algae organism. In order to prevent the cell membrane from fracturing in cold temperatures, cold-tolerant extremophiles may have adapted their lipid structure to maintain fluidity of the membrane. Bacterial research shows that membrane lipids do adapt to maintain fluidity in colder temperatures (5, 8, 9). A fuel produced from these extremophilic organisms may have a reduced melting point which would prove favorable for biodiesel distributed in colder climates.

Lastly, for naval applications, lipids from algal organisms are prepared differently for use in ships and planes than as biodiesel fuel for trucks. Due to the fact that ship fuel is kept in constant contact with seawater, algal lipids are force-hydrogenated once they are extracted from the organism. Force-hydrogenation removes all unsaturations and the ester and carboxyl group at the end of the fatty acid which creates fuel products such as Hydrogenated Renewable Jet (HRJ) fuel. Therefore, while analysis of fatty acid composition is important for commercial applications, the Navy is primarily looking for a biofuel feedstock that produces a high lipid by mass yield. Assessing the energy input in terms of growth rate, growth yield, and amount of natural resources consumed versus the energy output in terms of lipid size and yield is important not only for biodiesel fatty acid methyl esters but also for these hydrogenated alkane products used in ships and aircraft. Biodiesel and even raw algal oils are still of interest to the military as fuel options for land vehicles like tanks and trucks (10).

METHODS

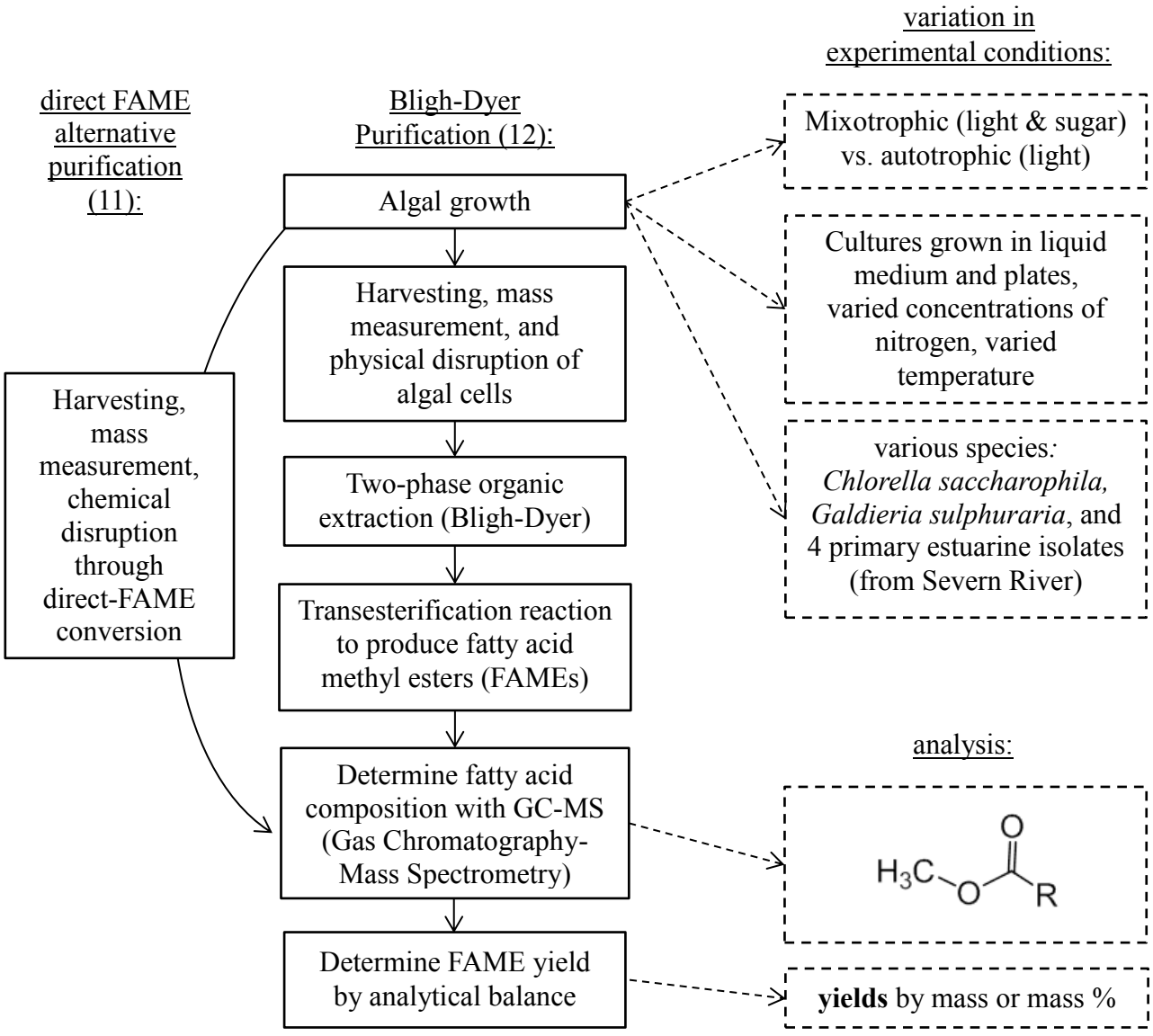
Objective: Investigate the lipid content and composition of extremophilic and estuarine microalgae for suitability as biofuel feedstocks and assess the impact of varying growth conditions on lipid content and composition.

Hypothesis: Extremophilic and local estuarine algae have unique potential as biofuel feedstocks.

Research Plan: To characterize lipid composition through organic extraction and FAME GC-MS to assess the effects of varying sugar and nitrogen content on lipid production
NOTE: A summary of the experimental methods is included below. For complete experimental methods see **Appendix A**.

Experimental Methods Summary: Six species of algae were grown under a variety of different conditions in both liquid medium and on plates. The temperature of the growth environment and the amount of nitrogen and sugar in the growth medium was varied. The six species included *Chlorella* (a green alga that served as the standard), four cold-tolerant estuarine algae, and *Galdieria* (a heat-tolerant extremophile found in hot springs). These algae were grown to a maximum density and then harvested from the growth medium. Through a series of chemical reactions, the lipids were extracted from each of the algae species and converted to fatty acid methyl esters (FAMEs) through the transesterification reaction. Both the dry mass of the algae and the dry mass of the biodiesel (FAMEs) produced for each species grown under different growth conditions were determined in order to calculate the mass of biodiesel (FAMEs) produced per culture volume and also per dry mass of algae. After the fatty acid methyl esters were produced, they were examined using gas chromatography-mass spectrometry (GC-MS). The GC-MS instrument sorted the different fatty acid methyl esters each algae produced by their volatility. This instrument also has software that allows the user to compare the mass spectrum (fragmentation pattern) of each FAME component to a set of standards to assist in the identification process. The types and relative amounts of the fatty acid methyl esters were then determined for each species under each different growth condition.

Experimental Methods Schematic:



RESULTS

GC-MS Results for Standards

Two standard mixes of fatty acid methyl esters were analyzed with GC-MS in order to later identify the unknown fatty acid methyl ester components in each algae species. **Figure 1** is an example of the GC-MS spectra collected. The peaks in the standard BAME Mix GC-MS spectrum (**Figure 1**) as well as the standard 37 Component FAME Mix GC-MS spectrum (not shown) were each characterized by retention time and MS matching to library standards (NIST) using computer analysis software. Standard peak identities were assigned for these column conditions (see **Appendix A** for experimental details) based on the manufacturer's list of components. The number in the left hand corner indicates the maximum intensity for the spectrum. **Table 1a** and **Table 1b** show the correlation between the retention time (RT) and the assigned peak identity of the standards. These tables were then used as references when interpreting the GC-MS spectra for the algae samples such as the spectra for *Chlorella saccharophila* displayed as **Figure 2**. The GC-MS instrument parameters were held constant for these standard runs as well as the GC-MS runs for the different algal strains in order to allow for an accurate comparison of retention times and identification of experimental chromatogram peaks.

Table 2a characterizes the lipids in the 250 mL mixotrophic *Chlorella* sample grown in liquid culture to be C14, C16:1, C16, C18:2, C18:1, C18. Additionally, the commercial *Chlorella* contains two C15 variants and an iso-C16 variant. As determined by comparisons between **Table 2a** and **Table 2b**, the autotrophic culture appears to have greater variation in the C16 fatty acid. In **Table 2b**, the peaks at 10.467 and 10.67 are determined to be C16:1 fatty acids with different placement of the unsaturations. This accounts for their difference in retention time. Based on the retention times, peak 2 and 3 are predicted to be C16 fatty acids with higher order unsaturations. Neither of the standards (37C nor BAME) have polyunsaturated C16 fatty acids so these peaks in **Table 2b** could not be identified. The fatty acid composition does not appear to change when sugar is added to the growth medium for the mixotrophic *Chlorella* growth conditions.

Chlorella is used in this work as the standard of comparison for both the cold-tolerant and heat-tolerant species examined. The fact that *Chlorella* is a well-characterized, common biofuel feedstock and grows at ambient temperature makes it an optimal standard of comparison.

Figure 1. GC-MS Peaks for Standard BAME (Bacterial Acid Methyl Esters) Mix

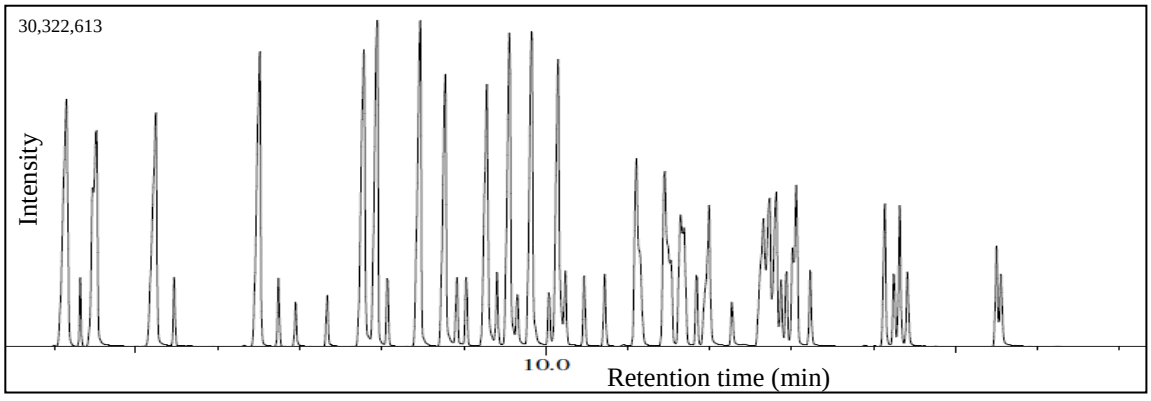


Table 1a: 37C FAME standard

Peak	RT(min)	ID
1	3.374	C10
2	4.324	C11
3	5.467	C12
4	6.734	C13
5	7.922	C14:1
6	8.067	C14
7	9.261	C15:1
8	9.403	C15
9	10.468	C16:1
10	10.719	C16
11	11.756	C17:1
12	11.995	C17
13	12.688	C18:3
14	12.946	C18:2(t)
15	13.229	C18
16	14.847	C20:4
17	14.927	C20:5
18	15.043	C20:3
19	15.270	C20:2
20	15.292	C20:1
21	15.565	C20
22	16.661	C21
23	16.995	C22:6
24	17.446	C22:1
25	17.721	C22
26	18.736	C23
27	19.502	C24:1
28	19.72	C24

Table 1b: BAME standard

Peak	RT(min)	ID
1	4.377	C11
2	4.531	2-OH C10
3	5.524	C12
4	6.792	C13
5	7.002	2-OH C12
6	7.385	3-OH C12
7	8.116	C14
8	8.961	i-C15
9	9.074	α-C15
10	9.451	C15
11	9.698	2-OH C14
12	10.082	3-OH C14
13	10.281	i-C16
16	11.569	i-C17
19	12.312	2-OH C16
20	12.911	C18:2
21	12.978	C18:1
22	13.048	C18:1
23	13.268	C18
24	14.285	C19:0$^{\Delta}$
26	15.594	C20

Figure 2. GC-MS Peaks for mixotrophic *Chlorella* from Liquid Culture

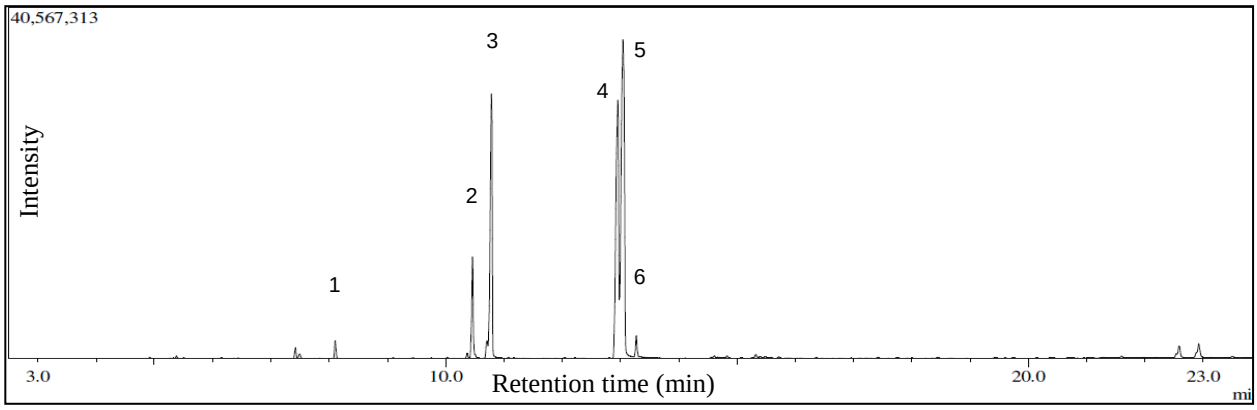


Table 2a. 250 mL Mixotrophic *Chlorella* from Liquid Culture

Peak	RT(min)	MS Assignment	ID (MS & RT Analysis)	Std RT	Reference
1	8.108	saturated FAME	C14	8.116	BAME
2	10.458		C16:1	10.511	BAME
3	10.773	saturated FAME	C16	10.761	BAME
4	12.946		C18:2	12.911	BAME
5	13.037		C18:1	13.048	BAME
6	13.273	saturated FAME	C18	13.268	BAME

Table 2b. 250 mL Autotrophic *Chlorella* from Liquid Culture

Peak	RT(min)	MS Assignment	ID (MS & RT Analysis)	Std RT	Reference
1	8.067	saturated FAME	C14	8.116	BAME
2	10.333		unsaturated C16		
3	10.415		unsaturated C16		
4	10.467		C16:1(7?)	10.511	BAME
5	10.67		C16:1(9?)	10.511	BAME
6	10.723	saturated FAME	C16	10.719	37 C
7	12.877		C18:2	12.911	BAME
8	12.966		C18:1	13.048	BAME
9	13.234		C18	13.268	BAME

Method Validation for New Direct-FAME Conversion

GC-MS Analysis: Hexane Layer

A new direct-FAME conversion procedure for converting wet algal pellets to FAMEs was developed by Chen in 2013 (11). For this procedure the wet algal pellet is reacted with a strong base and methanol in order to chemically lyse the cell and solubilize the hydrophobic material (to include the lipids). Then a strong acid is added to the solution which catalyzes the transesterification reaction, converting the lipids to fatty acids. This direct-FAME conversion procedure both extracts the lipids and converts them to FAMEs in one step instead of the two steps required for the old analytical method (a Bligh Dyer extraction coupled with a separate FAME conversion) (12). In order to test to see if the direct-FAME conversion was as effective as our previous method, both the old method and the new method were used to extract lipids from *Chlorella* which were then converted to FAMEs. **Figure 3** and **Figure 4** display the GC-MS spectrums for the FAMEs, found in the hexane layer, extracted through the two different conversion methods. The numbers above the peaks correlate with the table of fatty acids for mixotrophic *Chlorella*, **Table 2a**. The FAMEs should have all entered the hexane layer if all the lipids underwent the transesterification reaction. The fatty acids appear to be the same for both spectra. **Figure 4**, displaying the FAMEs in the hexane layer produced by the new method, is actually a cleaner spectrum with a greater signal. A minor C20 peak was also discernible in **Figure 4**.

Figure 3: *Chlorella* FAMEs produced through old method (Bligh Dyer then FAME conversion)

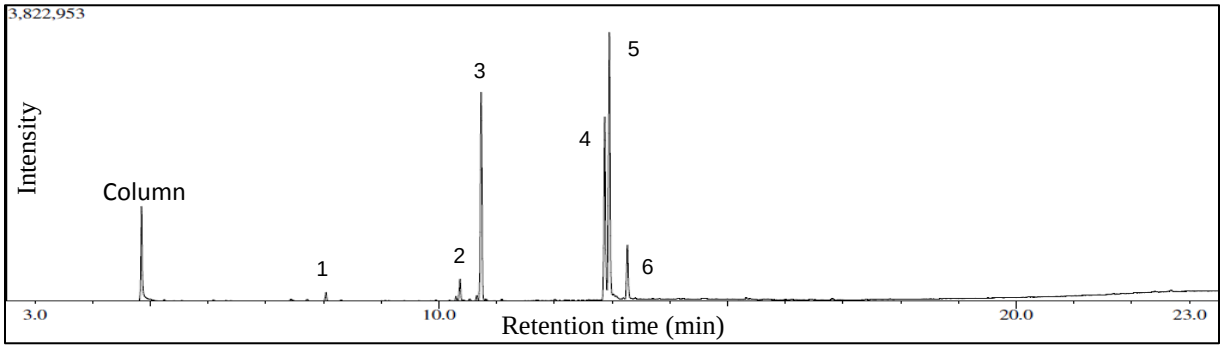
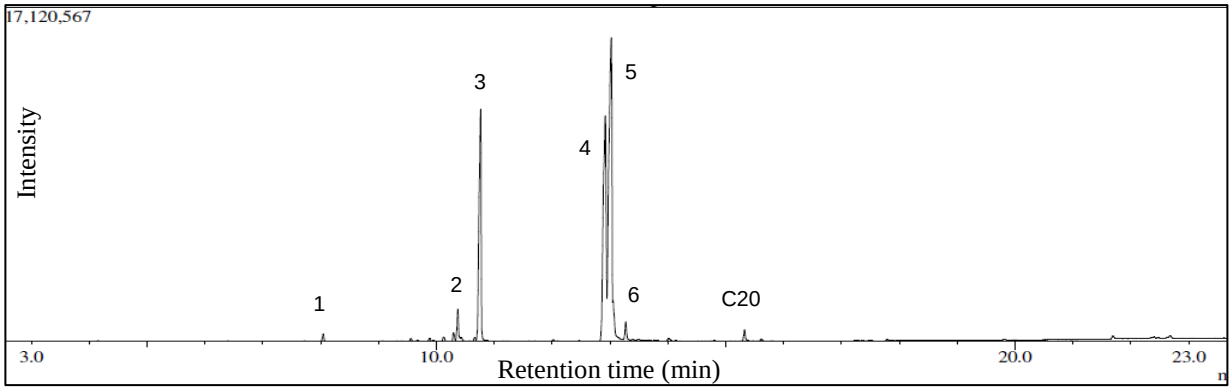


Figure 4: *Chlorella* FAMEs produced through new method (direct-FAME conversion)



GC-MS Analysis: Methanol Layer

Figure 5 and **Figure 6** display the spectrum for the FAME components found in the methanol layer after the transesterification reaction was completed. (The hexane was added to the methanol to extract the FAMEs, and the FAMEs should be completely soluble in the hexane layer). Interestingly, the FAME components observed in **Figure 3** and **Figure 4** were also seen in **Figure 5**, proving that the old method of converting algae into FAMEs does not solubilize all of the FAME components in the hexane. Instead, some of the FAME components remain in the methanol layer which results in lower FAME yields in the hexane layer. (The numbers above the peaks in **Figure 5** correlate with the table of fatty acids for mixotrophic *Chlorella*, **Table 2a**.) In contrast, there were no FAME peaks observed in the GC-MS spectra for the methanol layer after the new method was used to convert the *Chlorella* pellet to FAMEs. This suggests that close to 100% of the FAMEs produced through the direct-transesterification reaction are solubilized in the hexane layer as supported by the lack of peaks in **Figure 6**. The mass spectrometer was used to identify peaks in **Figure 5** and **Figure 6** as column components.

Figure 5: *Chlorella* FAMEs lost to methanol layer in BD extraction then transesterification

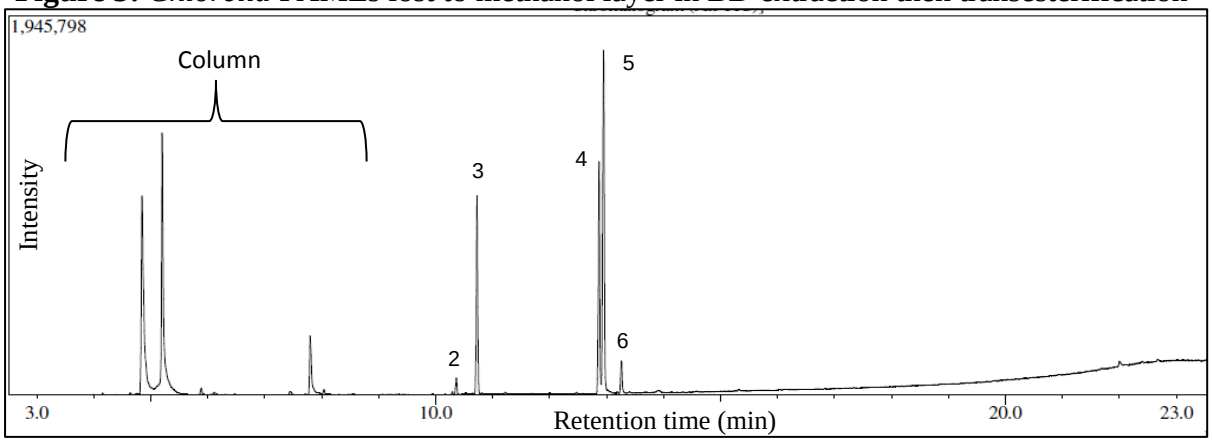
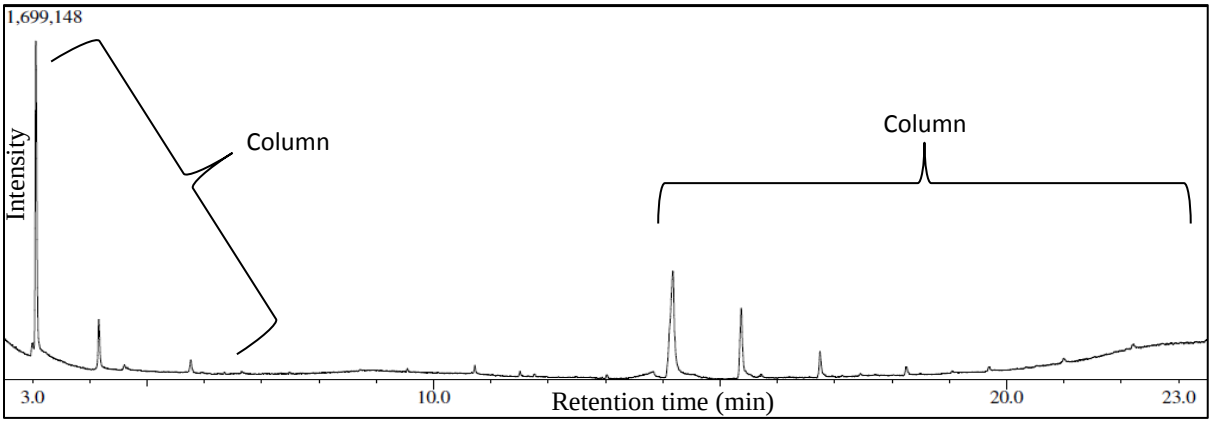


Figure 6: *Chlorella* FAMEs lost to methanol layer in direct transesterification



GC-MS Results for Extremophiles

Cold-tolerant species:

The FAME spectra for the algal strains obtained through winter bioprospecting of the Severn River are displayed in **Figure 7a** through **Figure 7d**. The three obtained specifically in College Creek (strains denoted CC) have similar fatty acid profiles with a higher degree of C16 and C18 unsaturation. In comparison, the Severn River Strain obtained from Santee Basin (denoted SB) does not have C16 unsaturation but has significantly more C16 than C18 in comparison to *Chlorella*. As seen in **Table 7**, C16 fatty acids with multiple unsaturations were identified, but their exact degree of unsaturation cannot be verified because, as they are unusual, the standard BAME and 37C mixes do not include polyunsaturated C16 fatty acids. The presence of these shorter chained (C16) and highly unsaturated fatty acids for the cold-tolerant species in comparison to *Chlorella* fits with the hypothesis that these strains would adapt their lipids in order to maintain fluidity at colder temperatures. CCe1("e1" indicates that this was the first strain cultured from College Creek) is of particular interest because this strain has an oily appearance on the agar plate, suggesting a higher lipid by mass ratio in comparison to the other organisms and/or the cell is eluting oils. Lastly, as seen in **Table 7**, there is still some uncertainty in classifying the C18 fatty acids for SBe1.

Figure 7a. GC-MS Peaks for Severn River Strain CCe1

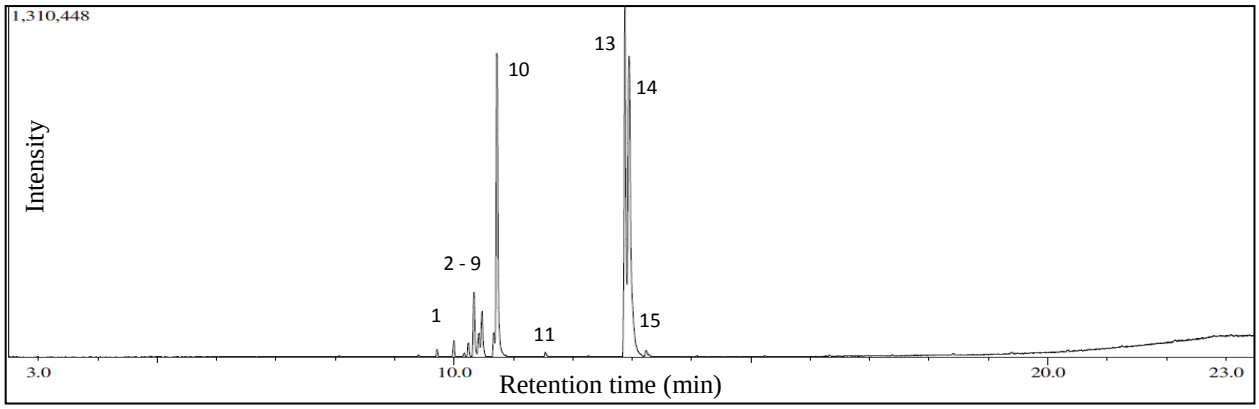


Figure 7b. GC-MS Peaks for Severn River Strain CCe2

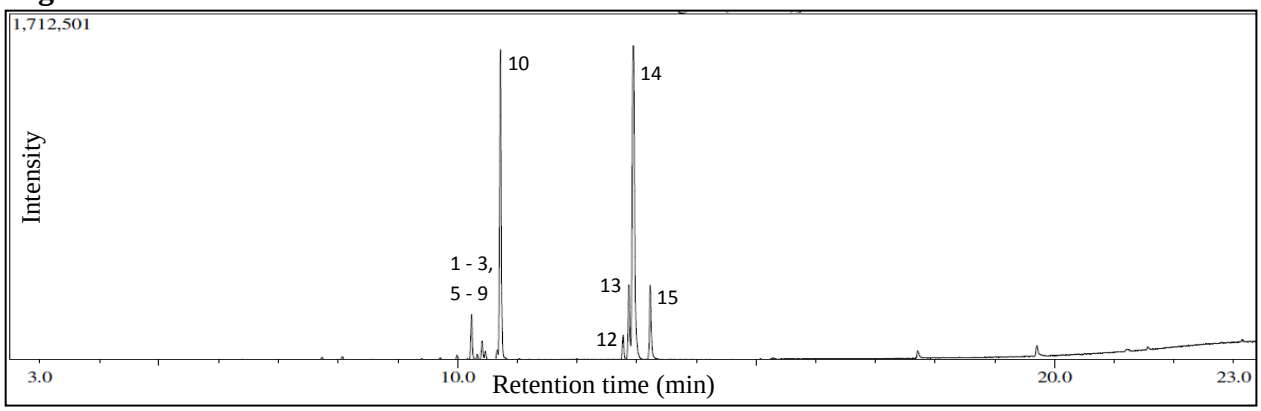


Figure 7c. GC-MS Peaks for Severn River Strain CCe9

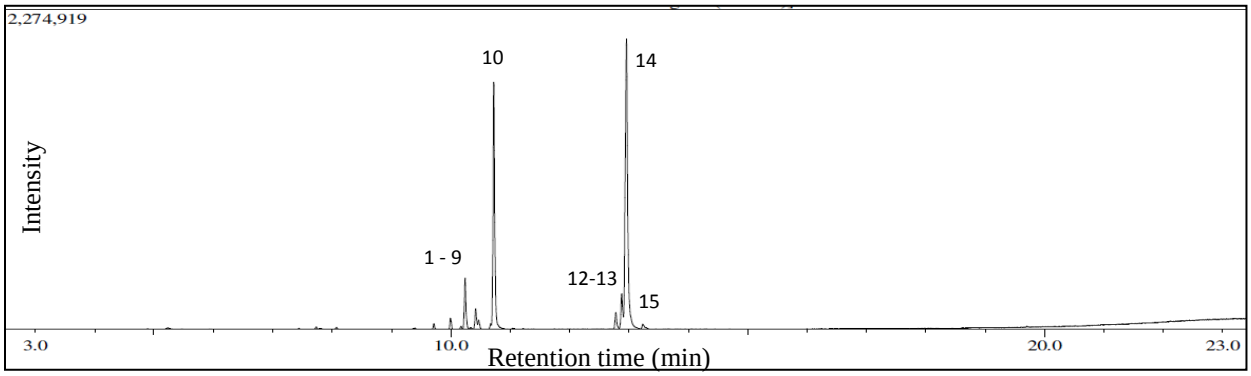


Figure 7d. GC-MS Peaks for Severn River Strain SBe1

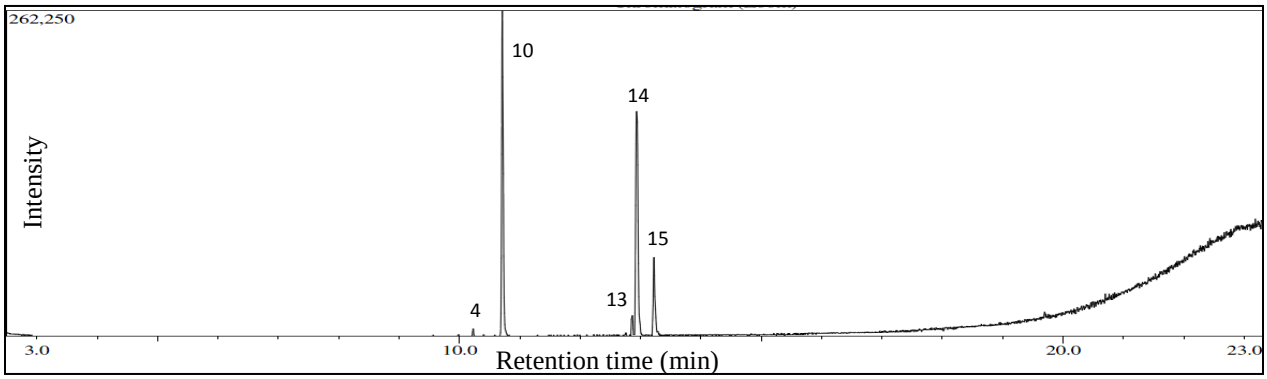


Table 7. Severn River Strains CCel, CCe2, CCe9, and SBe1 from Plate Culture

Peak	CCe1 peak (min)	CCe2 peak (min)	CCe9 peak (min)	SBe1 peak (min)	ID	Reference Std. (min)
1	9.4	9.407	9.433	---	C15	9.403
2	9.713	9.713	9.753	---	---	no ref.
3	9.993	9.993	10.033	---	---	no ref.
4	10.153	---	10.213	10.227	unsaturated, C16:3?	no ref.
5	10.233	10.233	10.273	---	unsaturated, C16:4?	no ref.
6	10.327	10.333	10.373	---	unsaturated	no ref.
7	10.413	10.413	10.453	---	unsaturated	no ref.
8	10.467	10.467	10.507	---	C16:1	10.468
9	10.66	10.667	10.707	---	unsaturated, C16:1?	no ref.
10	10.72	10.72	10.76	10.713	C16	10.719
11	11.533	---	---	---	i-C17	11.569
12	---	12.773	12.813	---	C18:3/C18:4?	no ref.
13	12.873	12.867	12.907	12.867	C18:2	12.911
14	12.953	12.947	12.987	12.94	C18:1	12.978
15	13.233	13.227	13.267	13.227	C18	13.268

Heat-tolerant species:

The *Galdieria* strains have proportionally more C18 fatty acids than C16 in comparison to the cold-tolerant strains, and *Galdieria* has no unsaturated C16 fatty acids which is an expected profile given its evolution for survival in hot environments. There appears to be no significant difference in fatty acid composition between the mixotrophic and the autotrophic sample, however, the autotrophic data were not as clean as the mixotrophic data, hence the presence of excess peaks which are simply gas chromatography column fragments. **Figures 8a** and **8b** and **Tables 8a** and **8b** detail the fatty acids in the *Galdieria* strain under different growth conditions.

Figure 8a. GC-MS Peaks for Mixotrophic *Galdieria*

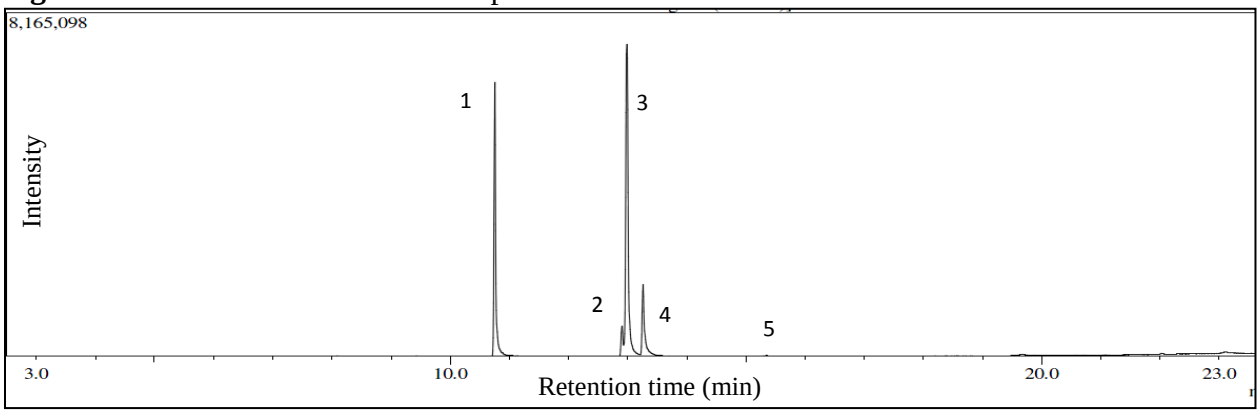


Figure 8b. GC-MS Peaks for Autotrophic *Galdieria*

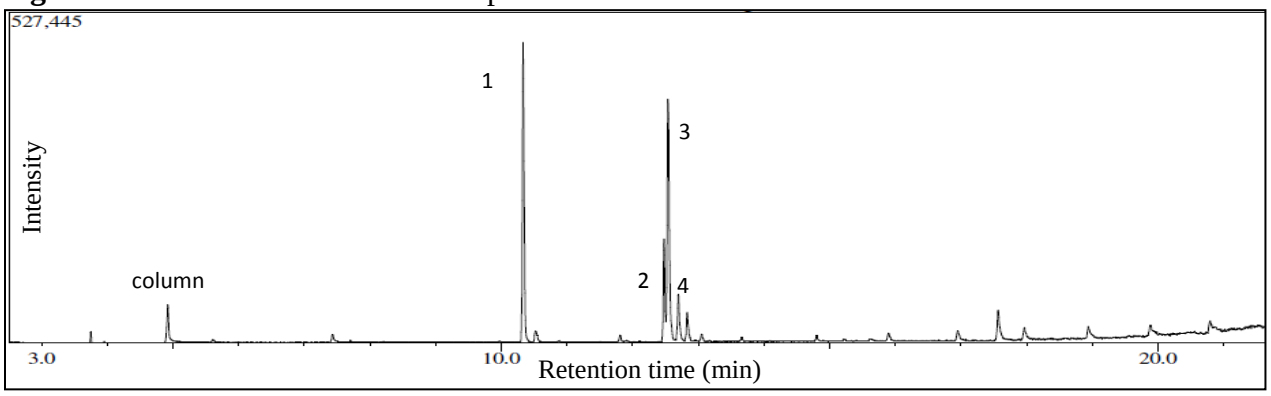


Table 8a. Mixotrophic *Galdieria* in Liquid Culture

Peak	RT(min)	MS Assignment	ID (MS & RT Analysis)	Std RT	Reference
1	10.753		C16	10.761	BAME
2	12.907	unsaturated	C18:2	12.911	BAME
3	12.993	unsaturated	C18:1	12.978	BAME
4	13.26		C18	13.268	BAME
5	15.347		C20:1?		37 C

Table 8b. Autotrophic *Galdieria* in Liquid Culture

Peak	RT(min)	MS Assignment	ID (MS & RT Analysis)	Std RT	Reference
1	10.340		C16	10.761	BAME
2	12.480	unsaturation	C18:2	12.911	BAME
3	12.540	unsaturation	C18:1	12.978	BAME
4	12.833		C18	13.268	BAME

Growth Conditions Testing

Optical Density Measurements

A Jasco V-550 spectrometer was used to measure the growth of *Chlorella* in liquid medium (**Figures 9a and 9b**) and *Galdieria* in liquid medium (**Figures 10a and 10b**) as optical density at 750 nm fixed wavelength in media with varying nitrogen content and the absence (autotrophic) or presence (mixotrophic) of 50 mM supplemental glucose. All raw data were normalized against the density of matched blank media samples, and densities of samples over 0.7 were assessed by 10-fold dilution in the appropriate medium to maintain readings in the linear range of the instrument. Both species grew poorly in media lacking nitrogen and reached similar autotrophic densities in nitrogen from 4.5 mM to 23 mM (standard) though mixotrophic cultures of *Chlorella* grew best in lower nitrogen (4.5 mM) than the standard 23 mM medium (this trend was not observed from *Galdieria*). Supplementation with sugar substantially increased both growth rate and maximum density. The optimum growth time for the mixotrophic cultures was determined to be one week, while 10 to 14 days growth for the autotrophic cultures was ideal.

Figure 9a: Optical density over time for mixotrophic *Chlorella* cultures with varied nitrogen

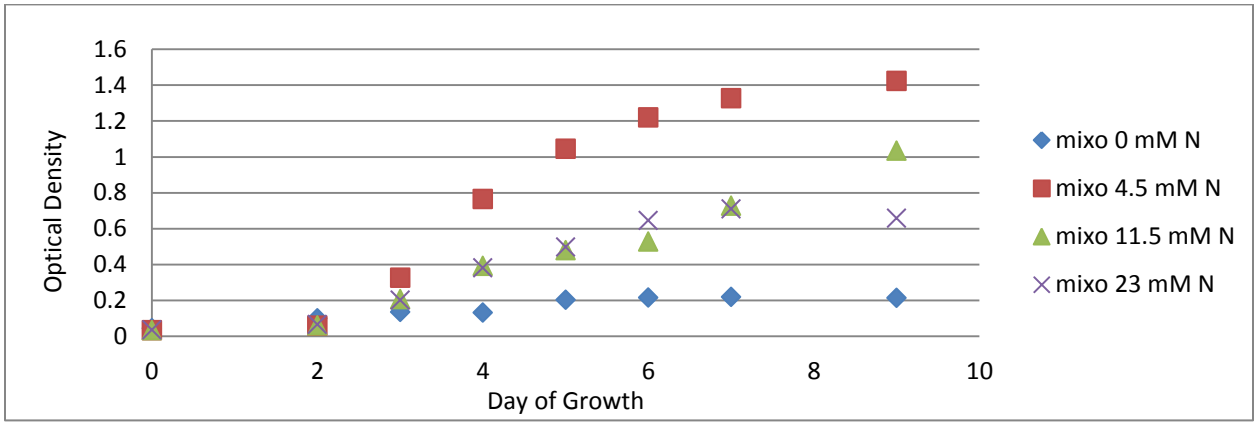


Figure 9b: Optical density over time for autotrophic *Chlorella* cultures with varied nitrogen

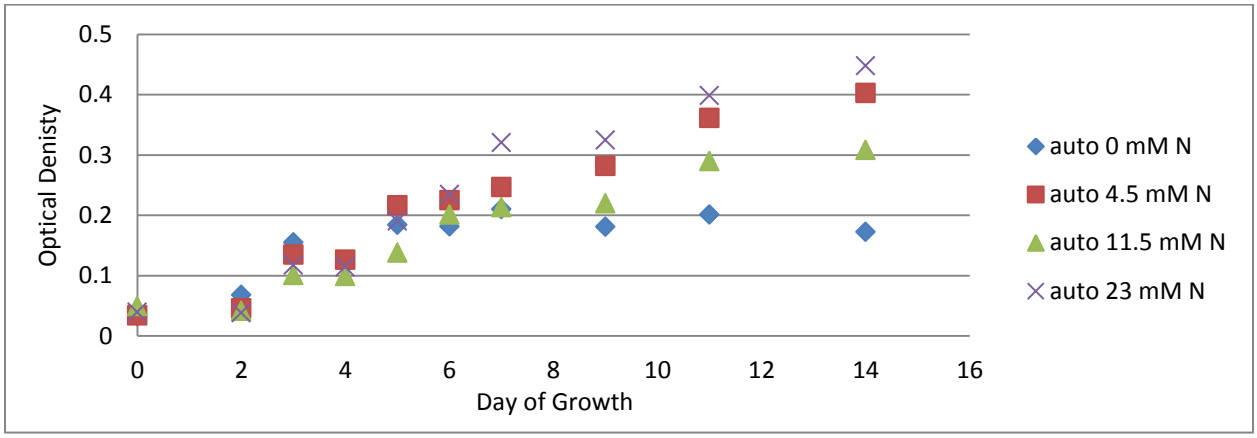


Figure 10a: Optical density over time for mixotrophic *Galdieria* cultures with varied nitrogen

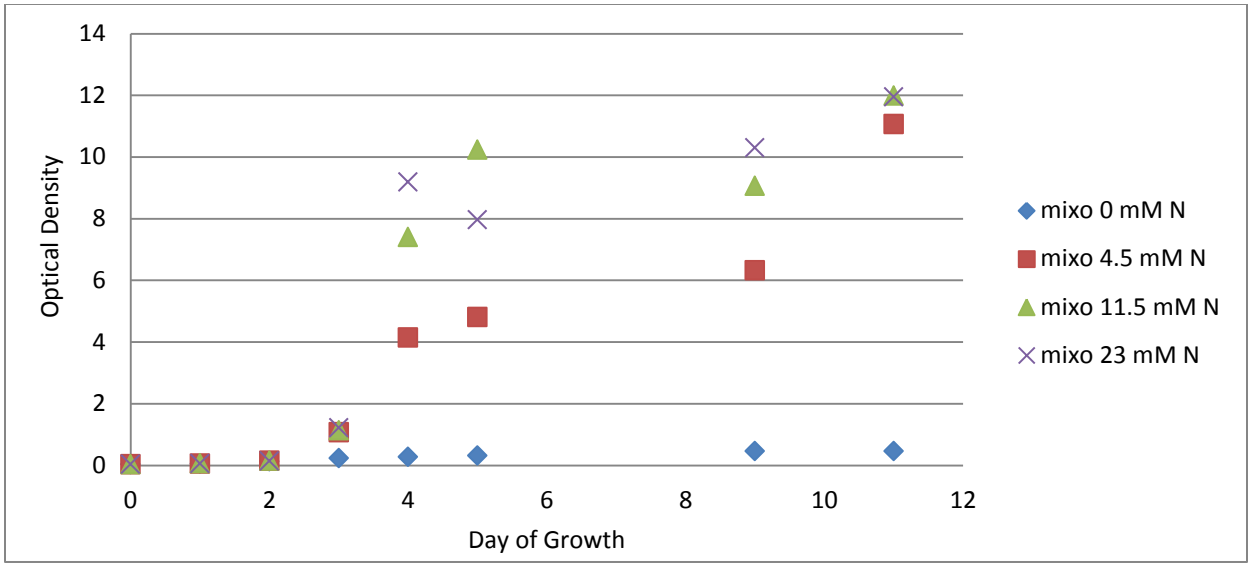
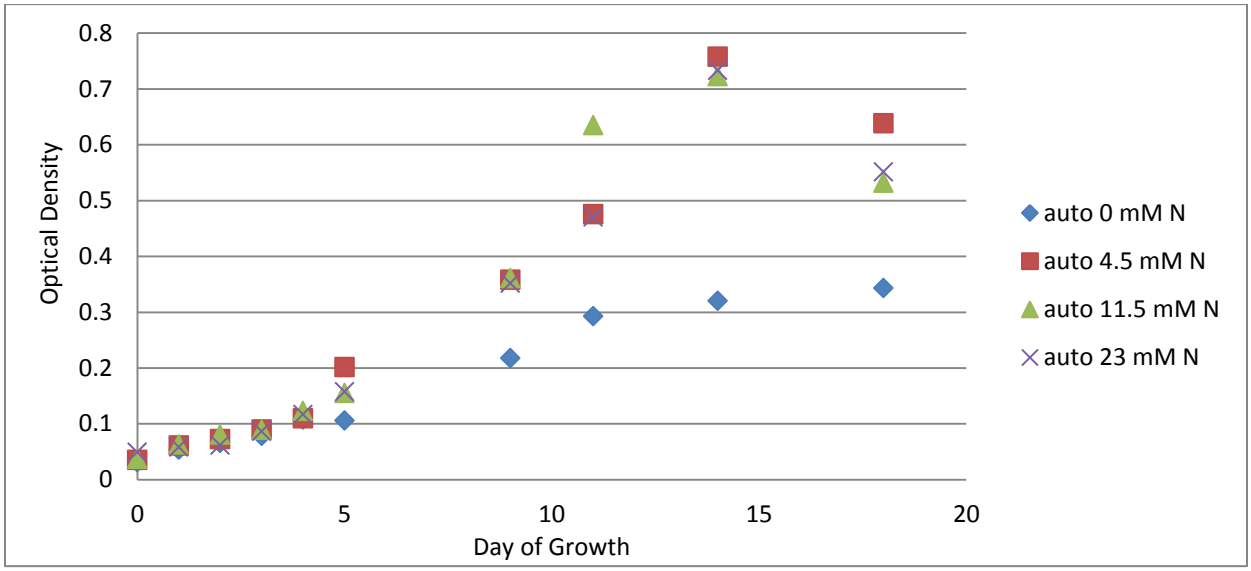


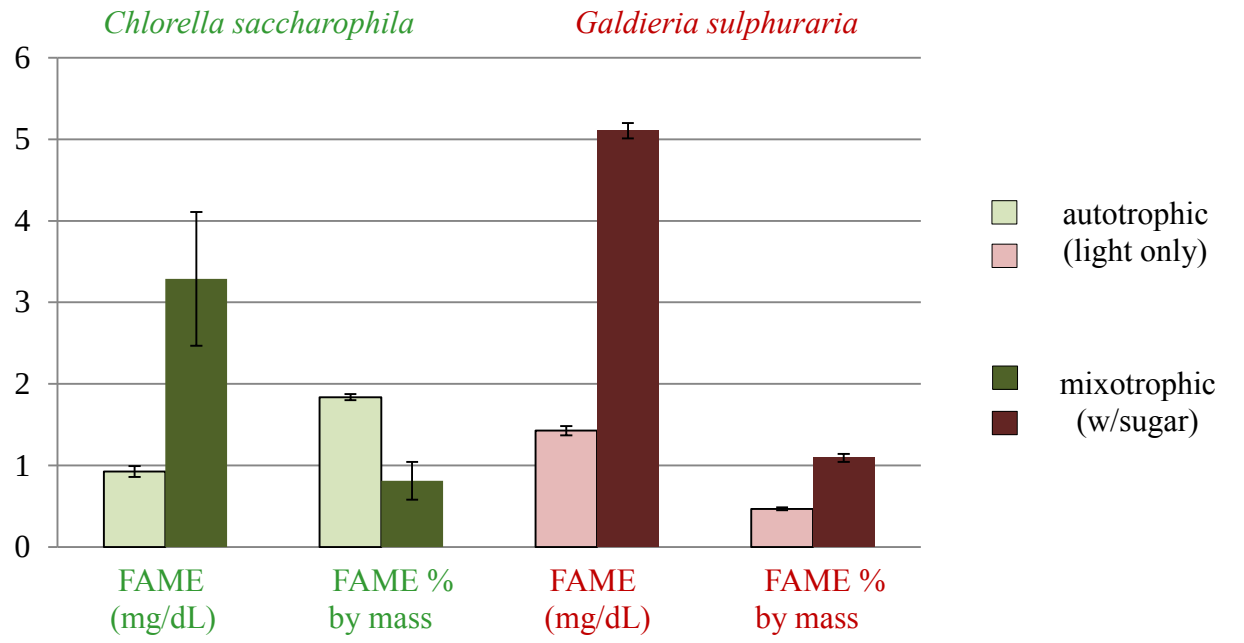
Figure 10b: Optical density over time for autotrophic *Galdieria* cultures with varied nitrogen



Total Fatty Acid Yield

Algal dry weight was determined using an analytical balance and tared bottles after lyophilization of total culture (250 mL) and normalized to mg/100 mL of culture. FAME dry weight was determined after direct extraction and drying the purified hexane phase. Two runs were completed and the averaged data are displayed in **Figure 11**. The error bars represent the calculated standard deviation of the series. *Chlorella* grew faster and to higher density when sugar was added to the growth medium for the mixotrophic runs (as supported by **Figures 9a** and **9b**), producing more absolute FAME mass per deciliter of solution but a smaller fatty acid percentage of total pellet mass; the sugar hindered the production of fatty acids. This trend in FAME mass and growth rate was also observed for *Galdieria*: with the addition of sugar *Galdieria* grows significantly faster as seen in **Figures 10a** and **10b** and produces more FAME mass per deciliter of solution. However, unexpectedly, when sugar was added to *Galdieria*, the percentage of total mass that is FAME was higher. This trend is the exact opposite of the *Chlorella* and other well-characterized feedstock algae (13). In addition to the absence of a heterotrophic carbon source, nitrogen deprivation has been reported to enhance lipid production in feedstock algae (13). In accordance with this, much effort was spent in this project examining the potential of nitrogen deprivation on lipid yield for *Chlorella* and *Galdieria*. However, the data from these nitrogen deprivation experiments have been omitted from this report as they have poor consistency and no repeatable positive or negative correlation between nitrogen content and lipid yield using these growth media and methods (data not shown). Additional experiments, perhaps with greater volumes of algal culture, will be necessary to address this potential contribution to feedstock optimization.

Figure 11: Fatty acid yields for autotrophic and mixotrophic *Chlorella* and *Galdieria*



FAME GC-MS profiles with varying nitrogen: Mixotrophic Samples

The lyophilized FAMEs for the *Galdieria* grown with varying amounts of nitrogen were profiled for fatty acid identity and composition by GC-MS in 2:1 C/M. **Figures 12a, 12b,** and **12c** display the spectra for the three mixotrophic *Galdieria* cultures of varying nitrogen amounts. These spectra show that varying the nitrogen content in the medium does not alter the fatty acids present or their relative proportions. The spectra looked the same for the autotrophic *Galdieria* samples as well (data not shown). The broad peak starting at around 15 minutes and continuing until 23 minutes (most visible in **Figure 12a**) is due to column degradation as a result of concomitant use of the instrument in teaching labs.

Figure 12a: *Galdieria* FAMEs in 0 mM N medium (FAME mass: 0.00273 g/ 166 mL culture)

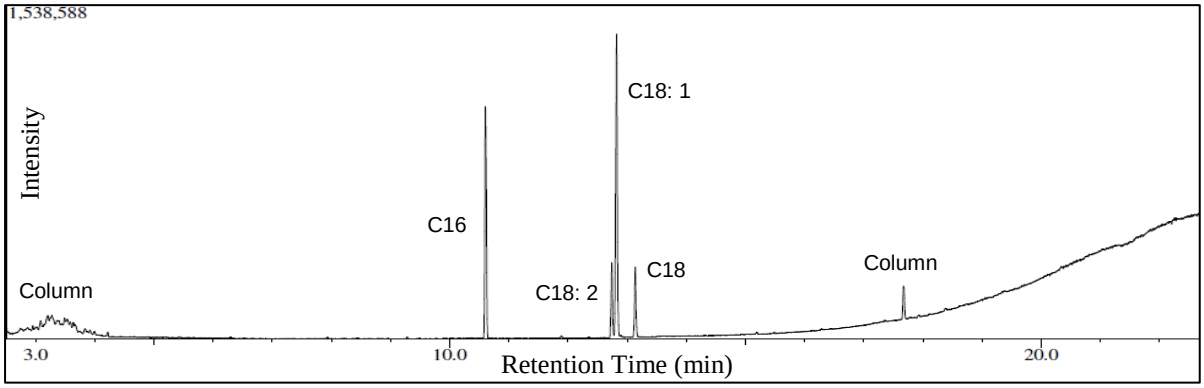


Figure 12b: *Galdieria* FAMEs in 4.5 mM N medium (FAME mass: 0.00430 g/ 166 mL culture)

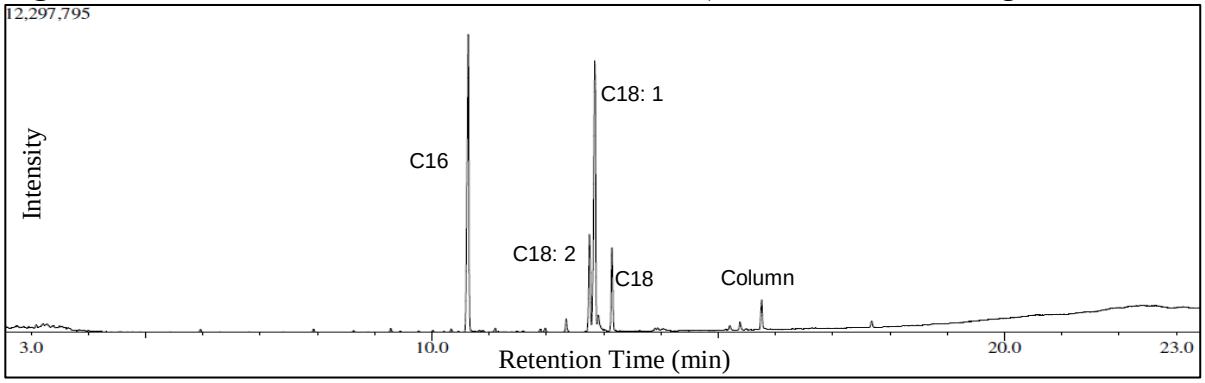
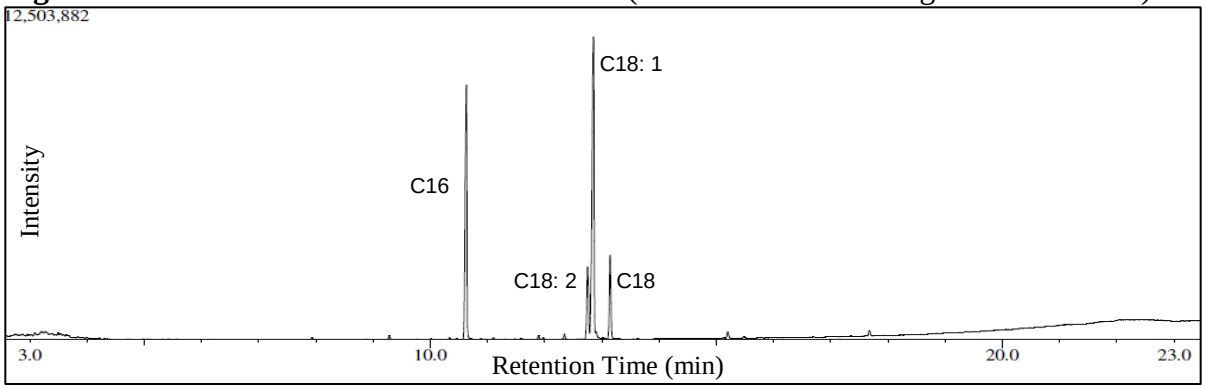


Figure 12c: *Galdieria* FAMEs in 23 N medium (FAME mass: 0.00563g/166 mL culture)



DISCUSSION

One of the most significant accomplishments of this research was the development of a procedure for effective growth, harvesting, and FAME extraction from extremophilic algal species. Over the course of this project, the experimental method flow chart included in this report was developed to optimize this effort, with a new direct-FAME alternative purification being the method of choice (**Figures 3** through **6** support this conclusion). This procedure proved superior to the traditional Bligh-Dyer extraction of microbial lipids, particularly for the extremophilic algae examined in this study. This procedure is effective and repeatable, and it will be used for further experiments into the suitability of extremophilic and estuarine microalgae as biofuel feedstocks.

In this project fatty acid-methyl ester analysis (FAME GC-MS) was used to characterize the lipid composition of both the well-known alga *Chlorella,* the unusual acidothermophilic alga *Galdieria sulphuraria*, and four cold-tolerant extremophiles harvested from the Severn River. The sugar and nitrogen content of the *Chlorella* and *Galdieria* growth media were varied in order to optimize algae growth rate and FAME production. In comparison to the typical algal fatty acids seen in *Chlorella*, the cold-tolerant extremophiles (CCe1, CCe2, and CCe9) have a significant amount of highly unsaturated C16 fatty acids. The CCe1 is a particularly interesting organism because it appears to have a high percentage of lipids to total mass. SBe1 has a proportionally greater amount of C16 fatty acids in comparison to C18. These species could potentially become biodiesel feedstocks that require less fuel additives in order to maintain fluidity. The heat-tolerant species, *Galdieria,* was found to have less unsaturation in the C16 fatty acids than *Chlorella,* as predicted, but greater lipid production by absolute mass and in ratio with total mass. The total lipid to pellet mass of this species in particular will be of interest to the Navy, which is primarily concerned with finding a biofuel that produces large quantities of oil for biodistillate production.

When the nitrogen content in the medium was varied for *Chlorella* and *Galdieria*, there was no discernible trend in the growth profiles (determined through optical density measurements) that was repeatable or coordinated with the total algal pellet mass or the FAME content. However, an unexpected trend was observed for the *Galdieria* organism when sugar was added to the medium. It is expected that when algae organisms are fed sugar (for a mixotrophic culture), the total pellet mass will increase due to the increased growth rate but the percentage of mass that is lipid will decrease. While this typical trend was observed for the *Chlorella* cultures, the *Galdieria* cultures responded differently to growth with sugar. Mixotrophic *Galdieria* medium containing 50 mM glucose as a carbon source increased algal growth as expected, but the FAME mass to total pellet mass also increased. In the past scientists have had to choose between accelerated growth rate and culture mass and increased percentage of lipids when deciding how to farm biofuel feedstock algae. The unusual ability of *Galdieria* to maintain or even increase relative production of lipids in mixotrophic medium could potentially be of great benefit in the development of algal biofuel production systems. This experiment will be repeated and conducted in greater volumes to verify and statistically analyze the results summarized in this report, and will also be extended to CCe1 and the other estuarine algae as lipid yield will be a crucial parameter of study in these as well. Nitrogen deprivation studies will also be repeated with a larger volume of medium in the hopes of improving the reliability of these data.

One of the aims of the original Trident Project proposal was to conduct fuels properties testing (to include melting point, fuel value, and viscosity) for the algal lipid products extracted from the different organisms. However, the focus of this project evolved into growth

optimization because in order to conduct the fuels properties testing, greater lipid yield is needed than was initially obtained. So far these experiments have not resulted in the extremely high oil yields that have been reported by some in the literature (15), however now that an effective and repeatable set of experimental conditions have been established, future work will focus on scaling up the growth and yield of these organisms to yield levels necessary for fuels properties testing.

CONCLUSION

This research project established the extent of oil content and the fatty acid composition of *Chlorella* and extremophilic algae (heat and cold tolerant organisms) under a variety of common growth conditions considered for biofuel feedstock production. The extremophiles produce some fatty acids that are distinct from those of common fuel feedstocks such as *Chlorella,* which may prove useful depending on the intended use and environment for the biofuel. In addition the hyperthermophilic alga, *Galdieria sulphuraria,* showed not only a distinct fatty acid profile but also an unusual response to supplementation with sugar, producing greater oil yield as a percentage of dry cell weight when grown with sugar rather than the lowered percentage yield seen in most algae. This characteristic, along with its heat-tolerant nature and capacity for extremely high-yield growth (6), makes *Galdieria* an attractive candidate for biofuels development.

ENDNOTES

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14. Umbach, B. "Characterization of microalgal lipids for optimization of biofuels" Division of Energy and Fuels paper number 323 in the Advances in Energy and Fuels Processes, Systems, Materials and Utilization session of ACS Dallas 2014.
15. Tang, *et al.*, *Biotech Bioeng* 2011, **108**(10), 2280-2287.

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APPENDIX A: DETAILED EXPERIMENTAL METHODS

1. Algal Growth

Chlorella and primary estuarine cultures (CCe.1, CCe.2, CCe.9, and SBe.1, isolated from the Severn River by ENS Erin D. Gehlhausen) were grown in Bold's Basal Medium (BBM) (autotrophic) or BBM with 20mM glucose (mixotrophic) at 20ºC with 16 hrs fluorescent light per day. BBM was produced as follows: 0.25 g $NaNO_3$, 4 mL of 0.075 g/mL $MgSO_4$ $7H_2O$, 10.0 mL NaCl, 10 mL K_2HPO_4, 10.0 mL KH_2PO_4, 10.0 mL $CaCl_2$ $2H_2O$, 1.0 mL H_3BO_3, 1.0 mL trace element solution, 1 mL of EDTA stock, and 1 mL of Fe solution. *Galdieria* was grown in Gs liquid medium with 20 mM glucose with constant fluorescent light (mixotrophic) or with no glucose and constant fluorescent light (autotrophic). *Galdieria* medium contained the following components per liter: 1.5 grams $(NH_4)_2SO_4$, 0.3 g $MgSO_4$ $7H_2O$ (0.147 g anhydrous), 0.3 g KH_2O_4, 0.02 g $CaCl_2$ $2H_2O$ (0.015g anhydrous), 1.5 mL Fe-EDTA stock solution, and 2.0 mL trace element stock solution. These components were brought to 1 liter with distilled water and the pH was adjusted to 1.8 by adding 1M H_2SO_4. Then the solution was autoclaved at 121˚C and ~15 psi (standards liquid cycle with no drying time). Liquid flasks and 15% agar plates of these media were autoclaved and prepared by standard microbiological procedures. *Chlorella* and the primary estuarine cultures were maintained on plates by serial monthly passage, and *Galdieria* by long-lived liquid culture, under the same conditions described above for the experimental cultures. For the BBM and *Galdieria* medium, the amount of nitrogen (in the form of $NaNO_3$ and $(NH_4)_2SO_4$, respectively) was varied to 50%, 20%, and 0% (11.5 mM, 4.5 mM, and 0 mM) of the original amount detailed in the procedure above (23 mM). Also, 2 mL of *Galdieria* parent culture were added per 167 mL of *Galdieria* medium and 2 mL of *Chlorella* or CCe1 parent culture were added per 167 mL of BBM medium. To the mixotrophic cultures, 4.2 mL of glucose (1M glucose 9g/50 mL) were added per 167 mL of liquid culture. The *Galdieria* samples (autotrophic and mixotrophic and all of all nitrogen levels) were grown in a hot room at 37ºC.

Optical Density Testing

A Jasco V-550 spectrometer was set to fixed wavelength measurement with wavelength equal to 750 nm. Liquid cultures were removed from flask shakers and placed in the sterile hood. In the hood, the flasks were swirled and then sterile plastic pipets were used to transfer ~1.2 mL of each liquid culture to a plastic cuvette. These pipets were inverted to insure uniformity across the sample, and then they were placed in the spectrometer once all bubbles had settled in the sample. An optical density measurement was recorded. In the case of an optical density measurement greater than 0.7, a 10-fold dilution with *Galdieria* or BBM medium (species dependent) was completed and the optical density measurement was then repeated. At least three optical density measurements were recorded per sample, and the average of these numbers was recorded.

2a. Harvesting and physical disruption of algal cell culture

Liquid culture was harvested by centrifugation at 2600xg at 4ºC for at least 15 minutes. Homogenization and disruption were completed in 100 mL of PBS using a microfluidizer. The sample was run through three times at 20 psi and collected in an Erlenmeyer flask for extraction. The wet cell mass was then lyophilized (freeze-dried under vacuum) and weighed. This constitutes the cell mass for future lipid to total mass ratio calculations. Algal cell mass was also harvested from plate culture by physical removal with a sterile pipet tip. The cell mass was then resuspended in 10 mL of phosphate-buffered saline (PBS) and disrupted with a probe sonicator (power setting 7 with 15 second pulses), followed by Bligh-Dyer extraction as described below.

3. Bligh and Dyer Extraction (two-phase organic extraction)

To an Erlenmeyer flask containing 0.8 volumes of aqueous cellular suspension, 1 volume of chloroform and 2 volumes of methanol were added, shaken, and allowed to sit for 40 minutes as a single-phase 1:2:0.8 chloroform/methanol/aqueous (C/M/aq) Bligh-Dyer mixture. The extract was then centrifuged in Teflon tubes using a Sorvall Legend RT centrifuge using the following parameters: acceleration/deceleration: 9/9, 2600xg, 4°C, and 15 minutes. The supernatant material was then decanted into a clean flask while the algal cell mass was washed with buffered single phase. The washed pellet and the original supernatant were centrifuged again to clarify the supernatant and recover additional extract from the washing of the pellet. All of the resulting supernatant was collected and converted to two-phase 2:2:1.8 C/M/aq mixture. For instance, in a flask bearing 150 mL of single-phase extract supernatant, 40 mL of chloroform were added, the bottle was shaken, and then allowed to stand for 10 minutes. Next, 40 mL of water were added in the same way and the mixture was centrifuged for 5 minutes under the previous parameters to resolve the aqueous and organic phases. The upper (aqueous) phase was then removed by decanting and by pipet, and the lower phase was washed with a volume of pre-equilibrated upper phase equal to that of the phase that was removed. After shaking to wash the lower phase the mixture was again centrifuged for five minutes to separate phases, and the lower phases were combined in a bottle and dried using a Buchi R-215 Rotavapor rotory evaporator and the dried lipid extract stored at -80ºC in a glass vial.

Determination of Total Lipid Content by Mass

The lipid extract was dried in air or under vacuum then weighed using an analytical balance. This mass was then compared to the mass of the lyophilized whole-cell sample of equal culture volume. The result was expressed as the percentage of the cell mass that is lipid.

4. Conversion of Lipids to fatty acid methyl esters (FAME) biodiesel

To a portion of the purified algal lipid extract, 500 µL of methanolic 3N HCl (transesterification reagent) was added and then the tubes were placed in an 80°C water bath for a 16 hour incubation to transesterify all acyl residues of the lipids to fatty acid methyl esters (FAMEs). The FAMEs were purified by adding an equal volume of n-hexane to the glass vial. The hexane layer (top) containing the FAMEs was extracted and this process was repeated at least two times to increase yield. The hexane layer was dried under nitrogen and stored at -80°C.

2b. Harvesting and Direct-FAME conversion (encompasses steps 2a. – 4. of alternative method)

Liquid culture was harvested by decanting the Erlenmeyer growth flasks into Teflon tubes. The Teflon tube samples were then lyophilized in order to obtain a dry pellet total mass. The algal pellets were suspended in 50 mL of 1M KOH in methanol. The solution was incubated for 15 minutes at 80°C with occasional venting. The bottle contents were cooled to room temperature and then centrifuged at 2600xg for 15 minutes. The supernatant was then recovered by decanting. To the supernatant, 1.5 mL of concentrated sulfuric acid was added, and then the solution was incubated for 15 minutes at 80°C minutes with occasional venting. The solution was left to cool to room temperature and then centrifuged at 2600xg for 15 minutes. The supernatant once again was recovered and then mixed with 50 mL of hexane. The top hexane layer was recovered which contained the FAMEs. The rotary evaporator was used to vaporize the hexane layer with the FAMEs remaining. The FAMEs were recovered with a 4:1 C/M extraction, transferred to a tared vial, dried under nitrogen, and then weighed in order to determine the FAME mass. The samples were then resuspended in 2:1 C/M for GC testing.

5. Analysis of fatty acid content (FAME GC-MS)

The FAMEs were dissolved in a small volume (~100 µL) of 2:1 chloroform/methanol for GC-MS analysis on 5% phenyl/95% dimethyl siloxane (30m SHRXI-5ms) using a Shimadzu 2010-SE GC-MS, 175-300˚C ramp at 10˚C/min with EI-MS detection. The EI-MS spectrum of each peak was used for preliminary peak identification through automated MS library matching. Further identification analysis was completed through comparing the retention times and spectra of authentic standard FAME mixtures from Sigma-Aldrich (BAME standard mix, 37-component FAME standard mix, and C8-C22 FAME standard mix).